Brain-Specific Autoantibodies in the Plasma of Subjects with Autistic Spectrum Disorder

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ABSTRACT: Although autism spectrum disorder (ASD) is diagnosed on the basis of behavioral parameters, several studies have reported immune system abnormalities and suggest the possible role of autoimmunity in the pathogenesis of ASD. In this study we sought to assess the incidence of brain-specific autoantibodies in the plasma of children with autism (AU) compared to age-matched controls including, siblings without ASD, typically developing (TD) controls, and children with other developmental disabilities, but not autism (DD). Plasma from 172 individuals (AU, $n = 63$, median age: 43 months; TD controls, $n = 63$, median age: 48 months; siblings, $n = 25$, median age: 61 months; and DD controls, $n = 21$, median age: 38 months) was analyzed by Western blot for the presence of IgG antibodies against protein extracts from specific regions of the human adult brain including the hypothalamus and thalamus. The presence of a ~ 52 kDa MW band, in the plasma of subjects with AU, was detected with a significantly higher incidence when compared to plasma from TD controls (29% vs. 8%, $P = 0.0027$ and 30% vs. 11%, $P = 0.01$, in the thalamus and hypothalamus, respectively). Reactivity to three brain proteins (42–48 kDa MW), in particular in the hypothalamus, were observed with increased incidence in 37% of subjects with AU compared to 13% TD controls ($P = 0.004$). Multiple brain-specific autoantibodies are present at significantly higher frequency in children with AU. While the potential role of these autoantibodies in AU is currently unknown, their presence suggests a loss of self-tolerance to one or more neural antigens during early childhood.

KEYWORDS: autism; autoantibodies; human brain; hypothalamus; thalamus

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BACKGROUND

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental diseases characterized by impaired social interaction, communication skills, imagination, as well as restricted and stereotypic behaviors. This condition is manifested within the first 3 years of life and persists into adulthood. ASD describes a range of conditions varying in behavioral symptoms and severity including autism, Rett's disorder, and Asperger's syndrome that fall within a larger classification known as pervasive developmental disorders (PDD).[1,2] The prevalence of ASD has increased substantially over 40 years from 5 to >60/10,000,[3–6] affecting males four times more often than females. It remains unclear whether the substantial increase in the prevalence of ASD can be attributed to heightened awareness and changing diagnostic procedures rather than new environmental influences.

Genetic, environmental, and immunological influences have been implicated in the pathogenesis of ASD, though the etiology and pathology of this complex disorder remain largely unknown. Several studies have reported immune system abnormalities in ASD,[7] including reduced natural killer (NK) cell activity,[8] incomplete T cell activation,[9,10] reduced numbers of CD4+ T-helper cells,[7,11,12] a reduced CD4+/CD8+ T cell ratio,[12] and elevated levels of plasma proinflammatory cytokines, such as tumor necrosis factor (TNF)-α.[13,14]

Immunoglobulin (Ig) abnormalities have also been reported in subjects with ASD, with a reduction in IgG subclass levels as well as an apparent increase in IgE levels.[7] In contrast, Trajkovski *et al.* showed significantly higher plasma concentrations of IgG subclasses compared to their developmentally normal siblings. Additionally, they found a decrease in IgA in ASD, providing evidence of impaired humoral immunity that could lead to a reduction in gastrointestinal protection.[15] Indeed, in a subset of subjects with ASD, gastrointestinal (GI) symptoms, such as enterocolitis and lymphoid nodular hyperplasia, have been observed. Mucosal lymphocytes isolated from these subjects with ASD and GI symptoms showed increased inflammatory and decreased regulatory cytokines compared to controls with and without similar GI symptoms.[16]

Circulating autoantibodies to various neuronal antigens have been found at a higher frequency in ASD groups compared to controls. Some of the targets include serotonin receptors,[17] myelin basic protein (MBP),[18] glial fibrillary acidic protein (GFAP), neuron-axon filament protein (NAFP) and neurofilament proteins,[19] neuron-specific antigens,[20] antibrain endothelial cell proteins and neurotrophic factors,[21] cerebellar neurofilaments,[22] and nerve growth factor.[23]

Advances in neuroimaging research have shown consistent neurological abnormalities in subjects with ASD including brain enlargement, a decrease in the size and number of purkinje fibers cells as well as altered brain chemistry, circuitry, and electrophysiology.[24] Currently, the focus of many studies has been confined to the cerebellum and the limbic system. The study described

herein expands to include additional regions of the brain, such as the thalamus, a relay point for sensory processing to the cerebral cortex, and the hypothalamus, which plays a key role in controlling the autonomic nervous system, endocrine system, limbic system, and the immune system. Since the thalamus and hypothalamus are involved in various neurological functions associated with ASD, any aberrations in these brain regions may be of importance in explaining the pathology of autism.

In the past, small sample size and unclear diagnosis have hindered the success of unraveling clear, conclusive pathogenic mechanisms involved in autism. This problem is further confounded by a lack of age-matched patient and control populations used in many of these studies. While several studies have reported the presence of immune dysfunction and/or autoimmune processes in subjects with ASD, there is as yet little consensus as to the true nature of these abnormalities. We hypothesized that subjects with autism have defining patterns of autoreactivity against neural antigens. Therefore, we analyzed the incidence of brain-specific antibodies in the plasma of subjects with autism and compared them to age-matched typically developing controls (TD), siblings, and subjects with other developmental disabilities but not autism (DD).

MATERIALS AND METHODS

Subjects

This study examined 172 children, enrolled through the Medical Investigations of Neurodevelopmental Disorders (MIND) Institute Clinic. The MIND Clinic sample population consisted of children diagnosed with autism and their typically developing siblings, a control population of age-matched typically developing children as determined by medical record abstraction, and children with mental retardation or developmental disabilities without autism.

To confirm in detail the initial diagnosis, all children were assessed at the UC Davis MIND Institute. Autism and ASD were confirmed for all cases using the Autism Diagnostic Interview-Revised (ADI-R[25,26]) and the Autism Diagnostic Observation Schedule, modules 1, 2, and 3 (ADOS[27–30]). The ADI-R provides a standardized, semistructured interview and a diagnostic algorithm for the DSM-IV 2 and the ICD-10 definitions of autism (World Health Organization [WHO], 1992).[31] The ADOS is a semistructured, standardized assessment in which the researcher observes the social interaction, communication, play, and imaginative use of materials for children suspected of having ASD. Final autism case diagnosis was defined as meeting criteria on the communication, social, and repetitive behaviors domains of the ADI-R and scoring at or above the cutoff for autistic disorder on the ADOS module 1 or 2. The Social Communication Questionnaire was used to screen for behavioral and developmental characteristics of ASD among the subjects with developmental

disabilities and among the typically developing controls; children who scored above the screening cutoff[32] were fully assessed using the ADI-R and ADOS.

The study for autoantibody screening included blood samples obtained from 172 age-matched samples (63 subjects with autism, 63 age-matched TD controls, 25 siblings of subjects with autism, and 21 DD subjects).

Antibodies and Reagents

Human adult brain protein medleys of the thalamus and hypothalamus (BD Bioscience Clontech, Palo Alto, CA, USA) were used to screen for autoantibodies in the plasma. Human kidney was used as negative tissue control (BD Bioscience Clontech, Palo Alto, CA, USA). Horseradish peroxidase-conjugated goat anti-human IgG was used as a secondary antibody (Zymed, San Francisco, CA, USA), and SuperSignal Chemiluminescent Substrate was used to develop the blot (Pierce, Rockford, IL, USA).

Immunoblotting

To determine the incidence of plasma reactivity to human brain extracts, SDS-PAGE was performed using 12%Tris-HCL Mini Ready gels (Bio-Rad, Hercules, CA, USA). A final concentration of 300 μg/mL of adult human brain extracts from the thalamus and hypothalamus, extracts from human kidney, and 5 μL of magic mark protein standard were loaded into the gel and electrophoresed for 1 h and 30 min at a constant current of 30 mAmps. After gel electrophoresis, proteins were transferred at 30 volts overnight to a nitrocellulose membrane, dried, and stored in 4°C. For immunoassay, the membranes were blocked with 5% milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) for 1 h at room temperature. Blots were incubated in plasma from subjects with AU, TD controls, normal siblings, and DD subjects diluted 1:500 in 5% milk and PBS-Tween for 1 h at room temperature. They were washed five times for 5 min durations with PBST, followed by 1 h incubation with HRP-conjugated goat anti-human IgG diluted 1:10,000. To visualize the signal, blots were developed using a SuperSignal Chemiluminescent Substrate according to the manufacturer's instructions. Strips were imaged and analyzed using FluorChem 8900 imager and AlphaEaseFC imaging software. (Alpha Innotech Corporation, San Leandro, CA, USA). Since multiple blots were used in this project, a reference patient, who demonstrated reactivity to all bands noted in this study, was run as a control on each blot.

RF and Molecular Weight

Measurement of band R_f, the ratio of the distance migrated by the protein to that of the marker dye front, and molecular weights (MW) were normalized

to the IgG band for each sample. Bands were manually selected and the R_f and MW were automatically calculated using AlphaEase FC Imaging Software (AlphaInnotech). AlphaEase FC assumes the origin (R_f value = 0.00) is located at the top of the screen and the dye front (R_f value = 1.00) is at the bottom of the screen. Using these points as a frame of reference, AlphaEase calculates R_f values for the intermediate bands. The MW of the band was calculated based on the graph of the known marker bands. In addition to the MW, an R_f difference of (+/– 0.005) was used as a distinguishing factor between bands.

Statistics

The chi-square test was used to compare the presence of antibodies in subjects with autism and to the control groups (TD controls, siblings, and DD group). *P*-values <0.05 were considered statistically different. Statistical analysis was performed using InStat GraphPad Software (version 3.0a).

RESULTS

Immunoblotting

Using Western blot analysis, plasma samples from 172 individuals (63 children with autism, 63 TD controls, 25 sibling controls, and 21 children with DD) were analyzed for the presence of IgG antibodies against proteins in the thalamus and hypothalamus. Multiple band patterns of reactivity were readily observed in plasma from subjects with autism. In comparison, significantly fewer band patterns were noted in the plasma from age-matched control subjects. Of note, specific autoreactivity was detected against a band with an apparent molecular weight of ~ 52 kDa (K band), with a significantly higher incidence in the plasma from children with autism, in the thalamus (29%, FIG. 1A) and hypothalamus (30%, FIG. 1B), when compared to the typically developing controls (8% and 11%, respectively, TABLE 1). Similarly, intense reactivity to a triplet band pattern at apparent molecular weights of ~48 kDa (L band), ~46 kDa (M band), and ~42 kDa (N band) in the thalamus (29%) and hypothalamus (37%), was noted in plasma from subjects with autism compared to the plasma from typically developing controls (8% and 11%, respectively, TABLE 1). A number of selected subject plasma samples, based on autoreactivity to the specific brain regions, were tested against an extract from human kidney to determine brain specificity of the reactive plasma. The specific reactive bands were not noted in the kidney in any of the samples tested.

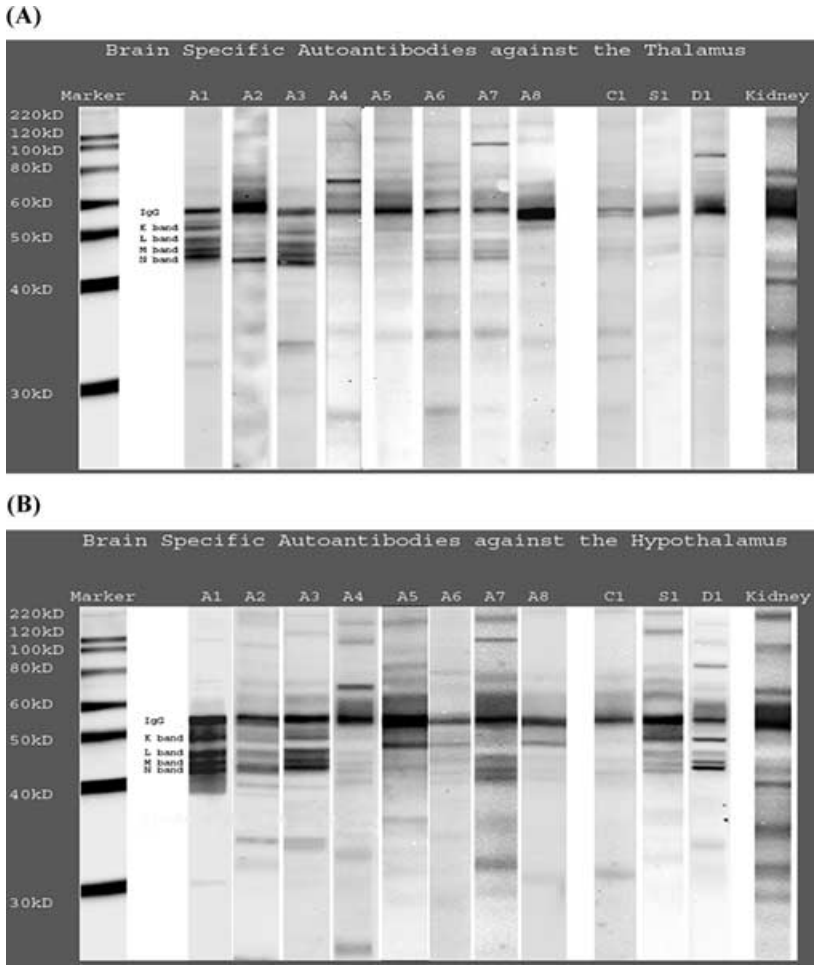

FIGURE 1. Immunoblotting of normal human adult brain. To illustrate the full range of band patterns, representative samples were used for FIGURE 1**(A,B)**. The same subjects were used in both A and B where subjects with autism are depicted in lanes A1–A9, while C1 represents a typically developing control, S1 represents a sibling control with some reactivity, and D1 represents a subject with developmental disabilities that demonstrates bands similar to the subjects with autism. **(A)** Immunoblotting of the thalamus has reactivity detected at approximately 52 kDa (K band), 48 kDa(L band), 46 kDa (M band), and 42 kDa (N band). As indicated in the figure, the detection of immunopositive bands was significantly higher with the plasma of ASD subjects compared to the plasma of TD controls, sibling, and DD subjects. The following six ASD subjects showed reactivity against the 52 kDa protein in the thalamus: A1, A3, A5, A6, A7, A8, but not in C1, S1, and D1. **(B)** Immunoblotting of the hypothalamus has reactivity detected at approximately 52 kDa (K band), 48 kDa (L band), 46 kDa (M band), 42 kDa (N band), and 35 kDa (P band). As indicated in the figure, immunopositive reactivity was detected with the plasma of ASD subjects, siblings, and DD subjects, but not with the plasma of the TD control subjects.

TABLE 1. Frequency of reactivity to a 52 kDa protein (K band) and a 42–48 kDa triplet band protein (LMN) in the thalamus and hypothalamus

	K Band (52 kDa)		LMN Band (42–48 kDa)	
Incidence (%)	Thalamus	Hypothalamus	Thalamus	Hypothalamus
AU($n = 63$)	18 (29%)*	19 (30%)*	18 (29%)*	23 (37%)*
TD($n = 63$)	5 (8%)	7 (11%)	5 (8%)	7 (11%)
Sib($n = 25$)	2 (8%)	6 (24%)	5 (20%)	7 (28%)
DD($n = 21$)	0 (0%)	2 (10%)	2 (10%)	5 (24%)
	K Band (52 kDa)		LMN Band (42–48 kDa)	
Significance (P-value)	Thalamus	Hypothalamus	Thalamus	Hypothalamus
Au vs. control	0.0027	0.017	0.014	0.0042
Au vs. Sibs	0.038	ns	ns	ns
Au vs. DD	0.0058	ns	ns	ns
Control vs. Sibs	ns	ns	ns	ns
Controls vs. DD	ns	ns	ns	ns

P-values indicate significant differences between the various sample groups. Note that the incidence of the bands, $P < 0.05$, in all the sections of the brain was significantly higher in the autistic compared to the typically developing control group.

DISCUSSION

This study analyzed plasma from subjects with autism for the presence of autoantibodies to human adult brain extracts by Western blot analysis. The results described herein demonstrated a significantly higher frequency of brain-specific autoantibodies occurring in children with autism compared to typically developing controls, siblings, and subjects with other developmental disabilities, but not autism (DD).

ASD is a highly heterogeneous disorder, and likely encompasses multiple subgroups of disease pathologies and etiologies. Thus, this complex disorder continues to baffle the research community and deciphering its etiology(ies) has proven extremely difficult. Small study sample size, unclear diagnosis, and unmatched populations have hindered the success of previous studies investigating immune factors in autism. Our comprehensive study, which includes well-defined population of age-matched subjects with autism, subjects with typical development, and disease controls corroborates previous reports of increased circulating autoantibodies in subjects with ASD.[17–23,33] In addition, our current findings further support the possible role of the immune system in the etiopathogenesis of at least a subset of subjects with ASD.

Concerns have been raised about the specificity of the circulating antibodies detected against various brain extracts. Previous studies have used very low dilutions of patient sera to probe specific brain extracts, which may have resulted in nonspecific binding. In this study, we addressed this concern by using

a higher dilution of subject plasma to increase the specificity and sensitivity of this assay. Additionally, the study described herein was performed using adult human brain extracts rather than rodent brain extracts to enhance species specificity.

The presence of circulating autoantibodies against brain-specific antigens in a subset of children with autism suggests potential autoimmune sequelae in at least some children with this disorder. Previous studies have reported putative autoantigens including MBP,[18] NFAP, and GFAP,[19] cerebellar neurofilaments,[22] neuron-specific antigens,[20] serotonin receptor,[17] endothelial cell proteins,[21] nerve growth factors,[23] and an as yet unidentified ~32 kDa protein found in human brain tissue extract.[33] However, with the exception of GFAP, the proteins of interest in this study do not correspond with previous findings according to the molecular weights of the intensely reactive bands noted herein. Moreover, when absorption studies were performed with purified GFAP, the 52 kDa band did not correspond to GFAP (data not shown).

There clearly remains a lack of consensus regarding the specific self-antigens recognized by autoantibodies in ASD. Thus, while it is possible that multiple antigens contribute to the etiopathogenesis of autism, it is also likely that there are numerous etiologies that lead to similar behavioral outcomes, and that autism is better termed "autisms." Moreover, it is not yet known which, if any, of the autoantibodies described in autism are pathogenic, and which are merely epiphenomena. However, even those autoantibodies that are not directly pathogenic provide us with potential clues as to the site of damage or injury to the brain of subjects with this neurodevelopmental disorder.

In the past, the central nervous system (CNS) has been viewed as an immunologically privileged site. This was due to the absence of conventional lymphatic drainage within the parenchyma and the presence of the restrictive blood–brain barrier (BBB) composed of unfenestrated endothelial cells with low pinocytic activity.[34] However, in areas of the brain including the pituitary, choroid plexus, subfornical organs, area postrema, and other circumventricular organs, fenestrations allow direct access to the CNS. Additionally, the CNS is subjected to immune surveillance by lymphocytes and resident cells, such as microglia and astrocytes.[35] In multiple sclerosis (MS), elevated levels of lymphocytes and antibodies in the CNS are associated with a compromised BBB. However, several studies have demonstrated that B cell trafficking occurs across a healthy BBB, which is capable of stimulating intrathecal antibody synthesis.[36] In a study by Stich *et al.*, anti-Yo intrathecal antibody synthesis was demonstrated in subjects with paraneoplastic neurological syndromes (PNS), which are associated with antitumor antibodies that cross-react with antigens in neuronal tissue.[37] In addition, activated T cells can enter the normal CNS despite antigen specificity or major histocompatibility complex (MHC) compatibility, but require both to remain in the brain.[38] Expansion of neural antigen-specific lymphocytes may occur in regional lymphoid tissues if the autoantigens are presented by antigen-presenting cells (APCs)

from the cerebral spinal fluid (CSF) and cervical lymphatics. These activated T and B cells could then potentially cross the BBB and elicit an immune response.

Antibody-mediated autoimmune disease has been reported in numerous neurological disorders. Schizophrenic subjects were reported to have elevated levels of antibodies to heat shock protein as well as antibrain antibodies in the CSF and serum.[39] MS subjects have been shown to have antibodies of all isotypes to autoantigens, such as MBP and proteolipid protein (PLP) in their CSF.[40–42] Antinuclear autoantibodies found in subjects with systemic lupus erythematosus (SLE) are known to contribute to several brain pathologies. However, it is only after the BBB has been compromised that these antibodies gain access to the brain ensuing neural damage.[43–45]

Prenatal immune dysregularities and the production of autoantibodies have been shown to alter the levels of cytokines, chemokines, neurotransmitters, and neuropeptides, influencing neural development.[46,47] Opponents of the immune dysregulation theory argue that the lack of an inflammatory response in the brain of children with autism negates the presence of autoantibodies and the possible role of the immune system in the pathogenesis of autism.[48] Recently, Vargas *et al.* demonstrated a marked increase in neuroglial cell activation and proinflammatory cytokine expression in the brain and CSF of subjects with autism. In addition to their role in the immune response, proinflammatory cytokines are capable of affecting the development and maintenance of the CNS.[49]

The presence of multiple brain-specific autoantibodies suggests that several neural antigens may be involved in the pathogenesis of autism. While we do not see these autoantibodies in all subjects with ASD, their presence may be associated with a subphenotype of children with autism and future studies will correlate the presence of specific autoantibodies and behavioral outcome. Further, the presence of autoantibodies to brain tissue found in a few typically developing children may represent a group of individuals who were not subject to sufficient genetic and/or environmental triggers for the expression of the disorder. The characterization of such brain-specific autoantigens is imperative to further define the role of autoantibodies in the etiopathogenesis of autism. Moreover, the results of this study may aid in developing a diagnostic tool for early detection of autism. Further study of immune dysregulation and the characterization of the autoantigen(s) involved will play a key role in deciphering the etiology of this spectrum of disorders.

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